Prevalence and Antibiotic Resistance Mechanisms of Methicillin-Resistant Staphylococcus aureus isolates from Selected Hospitals in Ashanti Region, Ghana.

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ABSTRACT

Background: Antibiotic resistance in bacteria has long been recognized as a major health problem occurring worldwide. The emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) remains a global health problem. MRSA is reported as one of the leading pathogens involved in increased rates of morbidity and mortality amongst patients in Ghana. This study determined the prevalence and resistance mechanisms of MRSA isolated from patients in selected hospitals in the Ashanti Region of Ghana. Antibiograms of the isolates were determined using the Kirby-Bauer disk diffusion method. Antibiotic resistance genes (*mecA and mecC*) were detected and efflux pump activity assessed using polymerase chain reaction (PCR) and microbroth dilution methods, respectively.

Results: Out of 626 samples obtained from patients, *S. aureus* was identified in 68, representing 10.9%. Multidrug resistance (MDR) was observed in 46 (67.6%) of the *S. aureus* isolates of which 28 (60.9%) were Methicillin Resistant *Staphylococcus aureus* (MRSA). The MRSA isolates showed higher susceptibility to trimethoprim-sulfamethoxazole (50%) and higher resistance to oxacillin and cefoxitin (100%). *mecA* gene was identified in 9 (32.1%) of the MRSA isolates whereas *mecC* gene was absent in all the isolates. The isolates did not exhibit any multiple efflux pump activities.

Conclusion: The prevalence of MDR-MRSA in *S. aureus* infections at healthcare facilities in the Ashanti region was found to be high. The presence of the *mecA* gene was identified as a possible mechanism responsible for resistance in the MRSA isolates.

Keywords: Methicillin-resistant *Staphylococcus aureus*, multidrug resistance, antibiotics, *mecA* gene, *mecC* gene, efflux pumps.

Background

Bacterial infections are a major cause of morbidity and mortality among humans all over the world (1). For several decades, antibiotics have increased the survival rate of patients (2). However, the emergence of resistant bacteria has become a significant problem within the health sector (3).

Staphylococcus aureus infection is reported as a menace in hospitals in most parts of the world (4). In Ghana, it has been reported as one of the most occurring MDR pathogen amongst patients reporting to regional and teaching hospitals for clinical care (2). The anterior nares and external skin surfaces of humans are frequently colonized by staphylococci causing skin and soft tissue infections and invasive infections (5).

Methicillin resistant *Staphylococcus aureus* (MRSA) is a strain resistant to methicillin and/or oxacillin (6). The occurrence and spread of MRSA in communities and health facilities are particularly worrisome as reports have indicated that MRSA is the leading pathogen involved in high rates of morbidity and mortality amongst infected patients (7).

The *mecA* gene in MRSA is responsible for the synthesis of an altered penicillin-binding protein, PBP2a, known to have a lower affinity for β-lactam antibiotics (8). Until the discovery of the *mecC* gene, detection of *mecA* in *S. aureus* was regarded as the gold standard for the diagnosis of MRSA infections (9). The gene sequence of mecC is about 70% identical to that of *mecA* and at the amino acid level, it is 63% identical to PBP2a. *MecC*-harboring *S. aureus* is often missed during phenotypic testing because it contains a type XI staphylococcal cassette chromosome with genetic makeup similar to oxacillin-susceptible MRSA (10), hence its detection requires genetic or molecular techniques.

Some studies have however reported of MRSA genotypes that possess neither *mecA* nor *C* genes; suggesting that other mechanisms could be involved in resistance to oxacillin and MRSA expression. Such mechanisms could include mutation in the PBP genes, hyper production of β-lactamases and multidrug efflux pump activity (10,11). In Ghana, very few studies have reported on the mechanisms responsible for resistance in MRSA. In addition, continuous monitoring of the prevalence and susceptibility patterns of MRSA in health facilities in Ghana is necessary for effective management and control of MRSA infections (12). Hence this study aimed to determine the prevalence and susceptibility patterns of *S. aureus* isolates including MRSA and identify possible mechanisms responsible for the resistance among the isolates in selected health facilities in Ghana.

RESULTS

Distribution of MRSA isolates amongst the hospitals

A total of 626 samples were collected from urine, wound and nasal swabs of patients visiting the selected hospitals. The samples were obtained from the hospitals as follows: 184 samples from Komfo Anokye Teaching Hospital (KATH), 163 from Agogo Presbyterian Hospital, 117 from Manhyia Government Hospital, 109 from Kwame Nkrumah University of Science and Technology (KNUST) Hospital and 53 samples from Suntreso Government Hospital (Table 1).

Based on growth on culture media, biochemical characteristics and SPA gene detection, a total of 68 (10.9%) *Staphylococcus aureus* strains were isolated from the 626 samples collected. Of the 68 *S. aureus* isolates, 46 (67.6%) were multi-drug resistant; non-

susceptible to at least one antibiotic in three or more antibacterial categories (13) (Table 1).

Seventeen (37.0%, n=17/46) of the MDR *S. aureus* were isolated from Agogo hospital, 14 (30.4%, n=14/46) from KATH, 13 (28.3%, n=13/46) from KNUST hospital and 1 (2.2%, n=1/46) each from Manhyia and Suntreso hospitals. Thirty-one (67%, n=31/46) of the MDR *S. aureus* isolates were identified from urine samples whereas 15 (32.6%, n=15/46) were obtained from nasal samples of the patients. None of the MDR *S. aureus* isolates was obtained from wound samples (Table 1).

Of the 68 *S. aureus* isolates, 28 (41.2%, n=28/68) were MRSA and all of these isolates were multidrug resistant. MRSA isolates were more prevalent in the samples from KNUST hospital (56.3%, n=9/16) compared to those from Agogo (48%, n=12/25) and KATH (31.8%, n=7/22). MRSA isolates were more prevalent in nasal samples (55.6%, n=10/18) compared to urine samples (36.7%, n=18/49). No MRSA isolate was obtained from wound swab samples (Table 1). MRSA isolates showed high resistance (60 to 100%) to all selected antibiotics and were more susceptible to trimethoprim-sulfamethoxazole (50%, n=14/28) (Table 1, Figure 1). MRSA isolates from KATH showed relatively low resistance to the different antibiotics compared to those from Agogo and KNUST hospitals (Table 1).

Six of the MRSA isolates were resistant to all the eight antibiotics tested. Erythromycin and ciprofloxacin showed relatively higher activity against the MRSA isolates from KATH whiles Trimethoprim-sulfamethoxazole was comparatively more active against

121 stains isolates from KNUST and Agogo. The most frequent patterns of resistance among
122 the MRSA isolates were ERY-OXA-CIP-CHL-SXT-TET-CN-FOX (21.4%) and ERY-
123 OXA-CIP-SXT-TET-CN-FOX (10.7%) (Table 2).
124
125 **Mechanism of resistance in multidrug resistant *MRSA* isolates**
126 **Presence of resistant genes (*mecA* and *mecC*) in multidrug resistant *S. aureus* strains**
127 Out of the twenty-eight (28) MRSA isolates, 9 (32.1%) had the *mecA* gene present. None
128 of the isolates possessed the *mecC* gene (Figure 2).
129
130 **Efflux pump activity amongst MDR MRSA isolates**
131 The MICs of ciprofloxacin against the isolates ranged from 0.8 to 12.8 μg/mL. In the
132 presence of reserpine, no reduction in the MICs were recorded. The MICs of ampicillin
133 against the isolates ranged from 3.2 to 12.8 μg/mL. Again, no reduction in the MICs were
134 recorded in the presence of reserpine, indicating the absence of multiple efflux pump
135 activity in the isolates.
136
137 **Discussion**
138 Increasing antibiotic resistance among pathogenic bacteria calls for more studies on the
139 prevalence and resistance mechanisms among these bacteria (1). Continuous monitoring
140 of prevalence, susceptibility patterns and resistance mechanisms of pathogenic bacteria,
141 including MRSA, in health facilities is also necessary for effective management and
142 control of bacterial infections (12).

Most of the reported surveillance on antimicrobial resistance of pathogens often involve samples obtained from large regional or teaching hospitals (2,12,14) with very few from smaller hospitals. In this study, samples were collected from a regional teaching hospital, four primary hospitals including one university hospital and three district hospitals in order to report on antimicrobial resistance at the district levels as well.

A review conducted by Bustamante (14) in 2011 indicated MRSA prevalence of 23 to 73% from different clinical samples across Europe, whereas in Ghana, some studies have reported prevalence of MRSA ranging from 28 to 34.8% (2,12,15,16). In this study, a prevalence of 41.2% was recorded. This is higher than that previously reported in Ghana. The difference could be due to the difference in sources of the samples.

Some studies define MRSA as *S. aureus* isolates resistant to oxacillin, possession of either *mecA* or *C* gene or a latex agglutination test for the detection of PBP2a (17–20). In this study, however, MRSA was defined as *S. aureus* isolates resistant to cefoxitin (30 µg, as suggested by the CLSI guidelines)(21) and such could lead to differences in the prevalence values observed. In as much as these assays are correct, over the years, the laboratory screening guidelines are updated and for example, oxacillin disk testing is no longer reliable as stated in the CLSI guidelines (21). Also, the specific detection of the mec genes or PBP2a could allow for some isolates to be missed. This is true because some studies have identified MRSA isolates which did not possess any of these genes and others had mutations, hence allowing for such isolates to be missed (11,22).

Most of the MDR MRSA isolates were identified in urine samples (18, 64.3%) compared to the nasal samples (10, 35.7%). However, 35.7% is of a relatively high prevalence in

nasal samples and this could lead to transmission to other patients at health care facilities as reported by Amissah *et al* (23). Also, performing nasal-swab screening could assist clinicians in tailoring down the choices of antibiotics to use (24).

The antibiograms of the MRSA isolates revealed an appreciable level of resistance to the test antibiotics (Tables 1 and 2, Figure 1). The isolates were mostly resistant to oxacillin and cefoxitin (100%), tetracycline (82.1%) and chloramphenicol (75%). Similarly, high resistance of MRSA to β-lactams (oxacillin) has been reported (12). Majority (50%) of the isolates were, however, susceptible to trimethoprim-sulfamethoxazole and this findings similar to that reported by Egyir *et al*, (25,26).

In this study, *mecA* gene was detected in nine (9) of the MRSA isolates (32.1%), representing approximately 13.2% of the total *S. aureus* isolates. Another study in Ghana reported a 28% prevalence of *mecA* genes in *S. aureus* strains isolated from various samples from patients at the Burns Unit of the Korle-Bu Teaching Hospital, Ghana (7) and this is higher than the 13% prevalence of *mecA* genes among patients in this study. The difference in *mecA* prevalence among the *S. aureus* isolates could probably be due to the fact that patients who suffer from burns and those on admission could be more susceptible to MRSA infections (27).

mecC genes were not detected in any of the *S. aureus* strains in this study. Globally, the prevalence of *mecC* genes in *S. aureus* is quite low; with a reported prevalence of

between 0.4% and 0.7% (28,29). The *mecC* genes have not yet been identified and reported in Ghana.

Apart from *mecA* and *mecC* genes, other factors such as the presence of efflux pumps have been identified to contribute to the development of resistance in *S. aureus* (30), hence the MICs of ciprofloxacin in the presence and absence of an efflux pump inhibitor (reserpine) was done to determine the presence of the efflux pump activities among the resistant isolates not containing the *mecA* gene. There was no change in the MIC of the ciprofloxacin and ampicillin in the presence of reserpine, suggesting very limited efflux activity in the evolution of MDR in the MRSA strains.

Among the 28 MRSA isolates, 19 (67.9%) had neither *mecA*, *mecC* nor enhanced efflux pumps activity. This shows that, other mechanisms may be responsible for resistance evolution in the MRSA isolates. Some studies have suggested mutations in the PBP genes, hyper production of β-lactamases and polymorphisms in the mec genes may be responsible for the resistance (31–33). There is a need to identify other possible mechanisms that may be responsible for the multidrug resistance in the *S. aureus* isolates especially in MRSA strains. This way, new drugs could be formulated and/or synthesized to tackle these pathways.

Conclusion

The prevalence of *S. aureus* isolates among the collected samples was 10.9% and 41.2% of these isolates were MRSA. The isolates were highly resistant to most of the reference

antibiotics except for trimethoprim-sulfamethoxazole. The presence of the *mecA* gene was identified as one of the mechanisms responsible for multidrug resistance in the *S. aureus* isolates. There is a need to continuously identity and monitor the level of resistance of *S. aureus* isolates in other health facilities and community acquired *S. aureus* infections in Ghana to enhance and improve the treatment of infections with *S. aureus* including MRSA.

Abbreviations

ATCC	American Type Culture Collection
CHRPE	Committee on Human Research, Publication, and Ethics
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic Acid
KATH	Komfo Anokye Teaching Hospital
KNUST	Kwame Nkrumah University of Science and Technology
MDR	Multidrug resistance
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSA	mannitol salt agar
MTT	3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
PBP	penicillin-binding protein
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
SPA	*Staphylococcus aureus* Protein A

Methods

Ethical consideration

The study was approved by the Committee on Human Research, Publication, and Ethics (CHRPE) the Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, with reference number CHRPE/AP/354/17. Permission and informed consent were sought from the various hospital authorities, subjects and their caregivers. In general, the study was performed in accordance with the Declaration of Helsinki (42).

Study sites

Five hospitals (Komfo Anokye Teaching Hospital, Kwame Nkrumah University of Science and Technology (KNUST) Hospital, Manhyia Government Hospital, Suntreso Government Hospital and Agogo Presbyterian Hospital), all located in the Ashanti Region, Ghana, were selected for the study. They comprised of one teaching hospital and four primary hospitals including a University hospital and 3 district hospitals.

Sample collection

Participants of the study were patients of all ages, who were suspected of having bacterial infections and were required to undergo clinical investigations at the microbiology laboratories, and patients who reported to the wound unit for wound dressing. Sterile cotton swabs were used to swab the anterior nares and wounds of the participants. The swabs were separately placed in 2 mL quantities of freshly prepared 10 mL sterile nutrient broth and labelled appropriately. Urine samples were collected into 30 mL sterile

containers and labelled appropriately. The samples were then transported with 12 h to the Microbiology Laboratory in the Department of Pharmaceutics, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology for analysis.

Isolation of *S. aureus* strains

Samples collected were placed into 10 mL nutrient broth and incubated at 37^{o}C for 24 h. After incubation, a loopful was streaked on 20 mL mannitol salt agar (MSA) plate. The inoculated plates were inverted and incubated at 37^{o}C for 24 h (34). Golden yellow colonies on MSA were aseptically fished out and inoculated into 10 mL nutrient broth and incubated at at 37^{o}C for 24 h. Gram staining and biochemical tests including hemolysis on blood agar, catalase and coagulase tests were performed on the 24 h broth culture to confirm the isolates as presumptive *S. aureus*, using *S. aureus* ATCC 25923 as the positive control organism (35).

Confirmation of Presumptive *S. aureus* isolates by Polymerase Chain Reaction (PCR)

The DNA of presumptive *S. aureus* isolates was extracted using the boiling lysis method as previously described by Meacham *et al*. (36) with some modifications. Single colonies of the presumptive *S. aureus* isolates, cultured on 20 mL nutrient agar plates, were transferred into 100 µL of phosphate-buffered saline (PBS), centrifuged at 15000 x g for 5 min at 25^{o}C and the supernatant discarded. One hundred microliters (100 µL) of PBS was then added, vortexed and the solution heated at 95^{o}C for 10 min and cooled at -20^{o}C

for 5 min. The mixture was again centrifuged at 15,000 x g for 5 min at 25^{o}C. The supernatant was collected into Eppendorf tubes and stored at -20^{o}C until used. Using the forward primer spa 1113-F (5'-TAA AGA CGA TCC TTC GGT GAGC-3') and reverse primer spa 1514-R (5'-CAG CAG TAG TGC CGT TTG CTT-3') (37), polymerase chain reaction was carried out using a thermal cycler (Gene Amp, ThermoFisher Scientific, Waltham, MA, USA USA). The PCR reaction was carried out in a final volume of 25 µL containing 2 µL of DNA template, 12.5 µL of One Taq master mix, 2µL (0.8 µM) of each primer and 6.5 µL of nuclease-free water. The PCR consisted of an initial denaturation at 94^{o}C for 5 min, followed by 35 cycles of denaturation at 94^{o}C for 30 sec, annealing at 60^{o}C for 1 min and extension at 72^{o}C for 1 min and a final extension at 72^{o}C for 10 min. The PCR products were separated on 1.5% $^{w}/_{v}$ agarose gel at 100V and visualized under UV light (16). Amplicon size ranged from 180 to 600 bp (38).

Antibiotic sensitivity testing

Antibiotic susceptibility of the *S. aureus* isolates was determined using the Kirby-Bauer agar disk diffusion method (39) following guidelines from the Clinical and Laboratory Standards Institute (CLSI) (21). A loopful of the confirmed *S. aureus* was streaked on 20 mL of nutrient agar and incubated at 37^{o}C for 24 h. Cells from five (5) to ten (10) well-isolated colonies were picked with an inoculating loop and emulsified into 2 mL sterile saline. The suspension was standardized to 0.5 McFarland either by diluting with more saline or addition of more bacteria cells. A sterile cotton-wool swab was dipped into the suspension, pressed firmly against the inner walls of the test tube to remove excess liquid, and then used to inoculate the surface of 20 mL Mueller-Hinton agar plate by continuous

swabbing of the plate while rotating at an angle of 60^{o} (approximately three times). With the aid of a disk dispenser, eight antibiotic disks from eight antibiotic classes including Erythromycin (ERY-15µg, Oxoid Ltd, Basingstoke, UK), Oxacillin (OXA-1µg, Oxoid Ltd, Basingstoke, UK), Ciprofloxacin (CIP-5µg, Oxoid Ltd, Basingstoke, UK), Chloramphenicol (CHL-30µg, Oxoid Ltd, Basingstoke, UK), Trimethoprim-sulfamethoxazole (SXT-25µg, Oxoid Ltd, Basingstoke, UK), Tetracycline (TET-30µg, Oxoid Ltd, Basingstoke, UK), Gentamycin (PIP-10µg, Oxoid Ltd, Basingstoke, UK), and cefoxitin (FOX-100µg, Oxoid Ltd, Basingstoke, UK) were delivered on the surface of the inoculated agar plate and incubated at 37°C for 18 h. Zones of growth inhibition were measured in millimeters using a millimeter rule. The experiment was carried out in triplicate and the mean zones of inhibition were compared to breakpoint values provided by the Clinical and Laboratory Standards Institute (CLSI) (21).

Detection of *mecA* and *mecC* antibiotic resistant genes in S. aureus.

PCR amplification of *mecA* and *mecC* genes was performed following procedures described by Boamah *et al*. (13) using the primers mecA-F (5‘-ACG GTA ACA TTG ATC GCA ACG-3‘), Mec A-R (5‘-GGC CAA TTC CAC ATT GTT TCG-3’) and mecCF (5‘-GAA AAA AAG GCT TAG AAC GCC TC-3’), mecC-R (5‘-GAA GAT CTT TTC CGT TTT CAG C-3’), respectively. Two microliters (2 µL) of DNA template was added to a final volume of 25 µL containing 12.5 µL of One Taq master mix, 2 µL (0.8 µM) of each primer and 6.5 µL of nuclease--free water. The PCR consisted of an initial denaturation at 94^{o}C for 5 min, followed by 35 cycles of denaturation at 94^{o}C for 30 sec, annealing at 59^{o}C and 55^{o}C for 1 min for mecA and mecC genes, respectively and

extension at 72°C for 1 min and a final extension at 72°C for 10 min. The PCR products were separated using a 1.5% $^{w}/_{v}$ agarose gel at 100 V and visualized under UV light. Amplicon sizes were detected at 176 and 138 bps, respectively.

Detection of efflux pump activity

The efflux pump activity of the *S. aureus* isolates was assessed using the efflux pump inhibition assay (41) utilising the microdilution method. The MICs of ampicillin and ciprofloxacin were determined in the presence and absence of reserpine using 96-well microtiter plates (40). The inoculum was prepared by suspending 5 to 7 well-isolated colonies in sterile normal saline to the density of 0.5 McFarland standard. Using a dilution factor of 2, different concentrations of the antibiotics were prepared from 0.8 to 12.8 µg/mL. One hundred microliters (100 µL) of double strength nutrient broth was dispensed into 5 wells. This was followed by the addition of appropriate volumes of ciprofloxacin for a given concentration (500 µg/mL) which was serially diluted by 2-fold into each well, followed by the introduction of 20 µL of the inoculum. Sterile water was then added to make a final volume of 200 µL. The plates were incubated for 24 h at 37°C after which 20 µL of 1.25 mg/mL 3-(4,5dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was added to the wells. Wells that showed purple colour after 30 min incubation at 37°C, indicated bacteria growth and those that remained yellowish indicated inhibitory activity. The MICs of ampicillin and ciprofloxacin were redetermined in the presence of 50 µg/mL concentration of the plant alkaloid reserpine (reserpine stock solution was prepared to 500 µg/mL).

Declarations

Ethical consideration

The study was approved by the Committee on Human Research, Publication, and Ethics (CHRPE) the Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, with reference number CHRPE/AP/354/17. Permission and informed consent were sought from the various hospital authorities, subjects and their caregivers. In general, the study was performed in accordance with the Declaration of Helsinki.

Data availability

All the necessary data on the above study is included in the manuscript.

Competing interests

The authors declare no competing interest in this study.

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Author contributions

CNZ carried out the laboratory exercises, data analysis and drafted the manuscript. VEB was involved in the conception, design, and coordination of the studies. YDB and HO participated in the data analysis and editing of the manuscript. CA participated in the

conception, experimental design and analysis of data. All the authors reviewed the manuscript.

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Figures

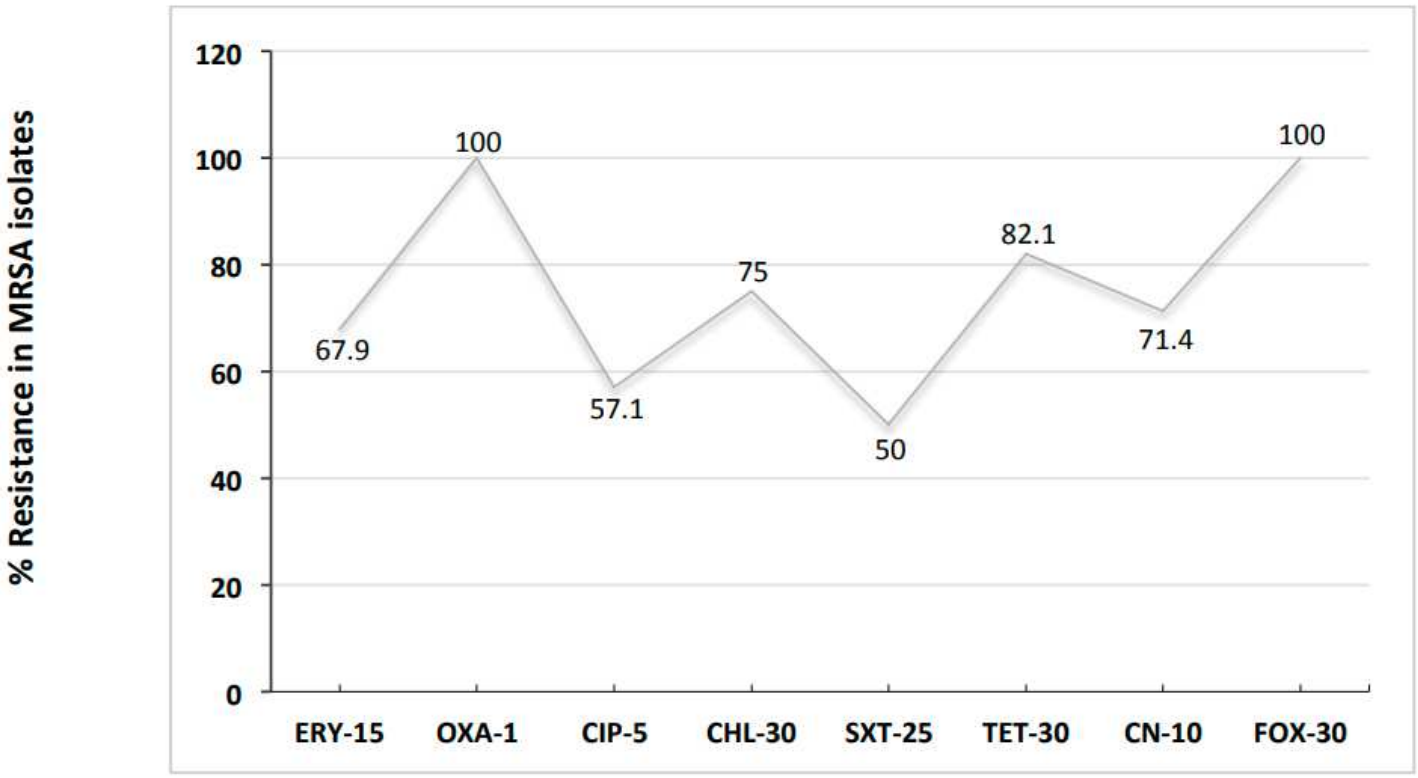

Figure 1

Antibiogram profile of the methicillin-resistant Staphylococcus aureus (MRSA) isolates. ERY-15: Erythromycin 15 µg; OXA-1: Oxacillin 1 µg; CIP-5: Ciprofloxacin 5 µg; CHL-30: Chloramphenicol 30 µg; SXT-25 Trimethoprim-sulphamethoxazole 25ug; TET-30: Tetracycline 30ug; CN-10: Gentamycin 10ug; FOX-30: Cefoxitin-30ug.

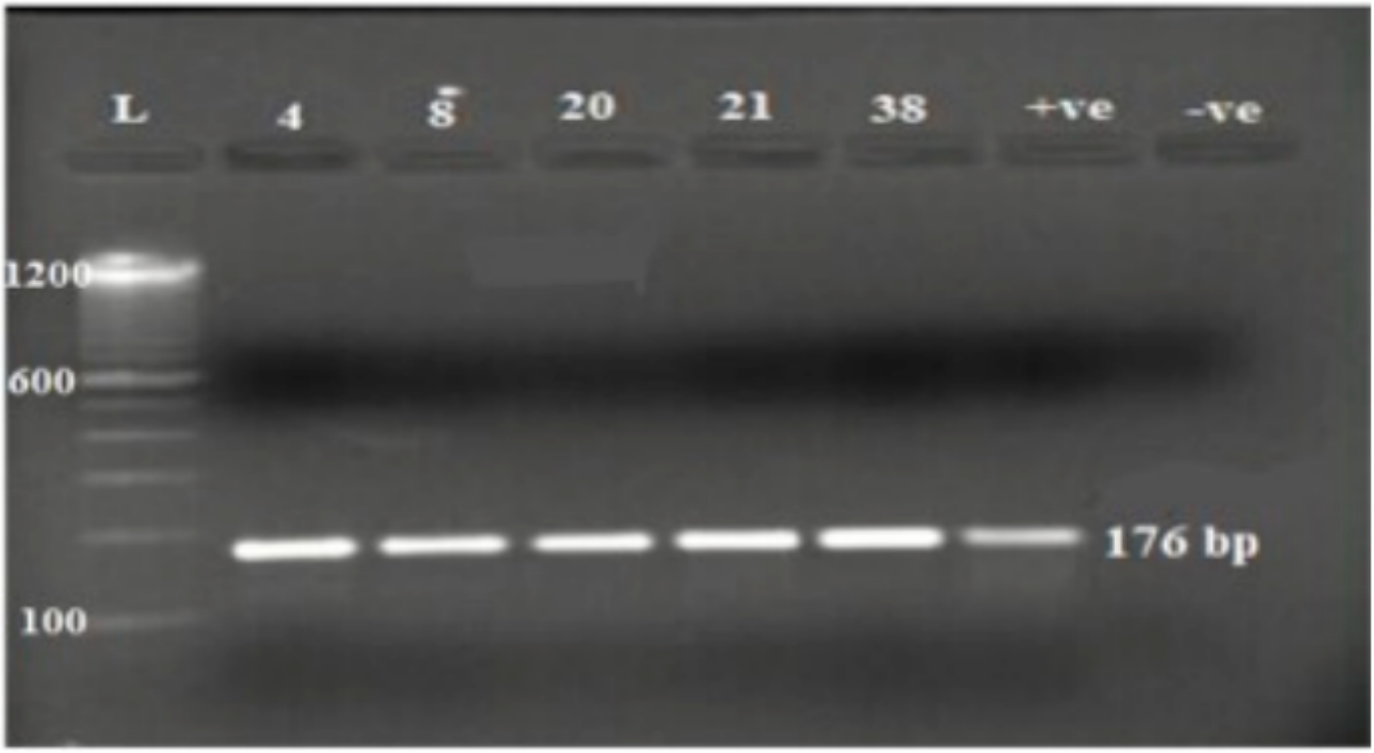

Figure 2

Electrophoretic gel image showing the 176 bp PCR amplicon of the mecA gene in MRSA isolates. L: 100 bp DNA ladder; +ve: Positive control (S. aureus USA 300); -ve: RNase□free water; 4 to 38; mec A positive S. aureus isolates

Supplementary Files

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- BMCtable1togo.pdf
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